

REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Application Serial No. 09/622,951, now allowed, filed August 24, 2000, which is the U.S. national phase of international patent application PCT/FR99/00453 filed March 1, 1999 and published as WO 99/44633 on September 10, 1999, which claims priority from French Application 0002800, filed March 3, 1998, each of which are incorporated herein in their entirety by reference.

Additionally, each of the applications and patents cited in this text, as well as each document or reference cited in each of the applications and patents (including during the prosecution of each issued patent; "application cited documents"), and each of the PCT and foreign applications or patents corresponding to and/or claiming priority from any of these applications and patents, and each of the documents cited or referenced in each of the application cited documents, are hereby expressly incorporated herein by reference, and may be employed in the practice of the invention. More generally, documents or references are cited in this text, either in a Reference List before the claims, or in the text itself; and, each of these documents or references ("herein cited references"), as well as each document or reference cited in each of the herein cited references (including any manufacturer's specifications, instructions, etc.), is hereby expressly incorporated herein by reference.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean, "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in greater detail with the aid of the embodiments taken by way of non-limiting examples and referring to the drawings.

Figure 1 depicts the nucleotide sequence of the EIV HA gene of the EIV Newmarket 2/93 strain.

Figure 2 depicts the nucleotide sequence of the feline herpesvirus-1 (FHV-1) gc gene.

Figure 3 depicts a graph showing the variation of viral excretion after experimental injection in horses vaccinated with the aid of different vaccines against EHV. In Figure 3, ■ represents vCP132 and water (A), ♦ represents vCP132 and carbomer (B), ▲ represents a commercial vaccine (C) and □ represents a control (D).

Figure 4 is a chart depicting the total clinical scores, including cough, dyspnea, nasal discharge, anorexia and general condition for both the vaccinated and control groups.

The present invention relates to an improvement to recombinant live vaccines integrating and expressing in vivo one or more heterologous genes. It relates in particular to such adjuvant-containing vaccines, to the
5 use of particular adjuvant compounds for using such vaccines as well as to vaccination methods relating thereto. Its subject is also a method of preparing these vaccines.

It is conventional to incorporate into
10 inactivated or subunit vaccines adjuvants intended to increase the immune response towards the antigens which these vaccines contain.

It has also been found to incorporate adjuvants into attenuated live vaccines when the attenuation of
15 microorganisms leads to a reduction in the immune response.

Recently, combined vaccinations against several pathogens using an inactivated vaccine for one valency and an attenuated live vaccine for the other valency
20 have also been proposed. It has thus been proposed to reconstitute the attenuated live vaccine, preserved in freeze-dried form, in the composition of an adjuvant-containing inactivated vaccine. The said composition acts as reconstitution vehicle for the live vaccine,
25 without any adjuvant effect being sought for it.

EP-A-532 833 proposes a vaccine against horse rhinopneumonia, a pathology caused by the equine herpesvirus (EHV). The vaccine is an inactivated and adjuvant-containing vaccine, grouping together the
30 inactivated EHV-4 virus and EHV-1 virus, containing the adjuvant Havlogen®, based on a polyacrylic polymer.

As for most herpesviruses, there is currently no effective vaccine allowing rapid elimination of the virus after infection. The known vaccines attempt to
35 protect against the appearance of clinical signs. In general, the effect on viral excretion remains limited.

According to EP-A-532 833, the vaccine developed is thought to lead to a drop in viral excretion ranging from 79 to 93% (see results section).
40 Eight control animals out of nine excreted virus after

a challenge over an average of 1.4 days whereas the normal duration of excretion after challenge is usually greater than or equal to 5 days. This represents a challenge of low intensity which artificially increases the protection of vaccinated horses compared with the controls. The reduction in viral excretion is not therefore significant in this experiment.

Adjuvants of the carbomer type have also been used in inactivated virus-containing equine influenza vaccines (IEV).

Mumford et al (Epidemiol. Infect. (1994), 112, 421-437) recall that two equine IEV vaccine doses are required to induce a transient humoral response and a weak protection against the virus in horses. The authors compare the adjuvant effects of carbomer and of aluminium phosphate on inactivated vaccines in the presence or otherwise of tetanus toxoid. In all cases, a low antibody titre measured by the SRH (single radial haemolysis) technique with respect to the strains H7N7 and H3N8 is obtained after a first vaccination and a second and then third vaccination are necessary to see the appearance of stronger transient responses.

US-A-4 500 513 also presents vaccination trials against the equine influenza virus with an inactivated vaccine in the presence of a carbomer. The origin of the animals and their medical status is not indicated precisely and it appears that they are ground animals (column 11, 2nd paragraph). The high antibody titres, measured by a haemagglutination inhibition technique, indicate that the animals had probably already been infected with influenza and that the response induced after vaccination was of the booster type, and not of the primary vaccination type.

Finally, Fort Dodge Solvay markets inactivated equine influenza vaccines (Duvaxyn® IE and IE-T plus) and an inactivated equine rhinopneumonia vaccine (Duvaxyn® EHV_{1,4}), in a carbomer adjuvant.

Commercial inactivated vaccines against equine influenza, containing the adjuvant aluminium

hydroxide (for example Tetagripiffa®, Merial, Lyons, France) are also known.

5 A large number of other adjuvants are used in the context of conventional inactivated or subunit vaccines. There may be mentioned, for example, aluminium hydroxide, aluminium phosphate, Avridine®, DDA, monophosphoryl lipid A, Pluronic L121 and other block polymers, muramyl peptides, saponins, trehalose dimycolate, copolymers of maleic anhydride and
10 ethylene, copolymers of styrene and acrylic or methacrylic acid, polyphosphazene, oily emulsions and the like.

WO-A-94 16681 suggests supplementing a recombinant live vaccine expressing a heterologous gene
15 of an enveloped virus with an adjuvant vaccine composition in the form of a water-in-oil, oil-in-water or water-in-oil-in-water emulsion.

Such a solution may however have a number of disadvantages.

20 In practice, the final user should have available, on the one hand, a freeze-dried active ingredient and, on the other hand, an already constituted emulsion which should make it possible to reconstitute the freeze-dried active ingredient.

25 Lack of stability of the emulsion during storage could be detrimental to the efficacy and safety of the reconstituted vaccine.

The activity of attenuated live microorganisms could be called into question following their
30 instability in the oily phase. This may in particular be the case for viruses which may thereby lose their viability.

Vaccines in emulsion can also pose problems of safety at the site of injection.

35 The present invention is therefore given with the objective of providing new vaccine compositions based on recombinant live vaccine expressing at least one heterologous nucleotide sequence, especially a heterologous gene, containing an adjuvant which is

capable of remarkably increasing the immunity conferred relative to the same vaccine with no adjuvant and which is perfectly suitable for this type of vaccine.

5 The Applicant has found the carbomer class of compounds were capable of acting as adjuvant under the required conditions for this type of vaccine and this in unexpected proportions. Trials carried out on animal herpesviruses (EHV-1, Equine Herpesvirus) have shown that the supply of carbomer could reduce viral
10 excretion during an experimental infection, in unexpected proportions. Other trials carried out on the equine influenza A virus have made it possible to obtain, surprisingly, in horses, early and very high serological titres, better than those obtained with the
15 best commercial vaccines.

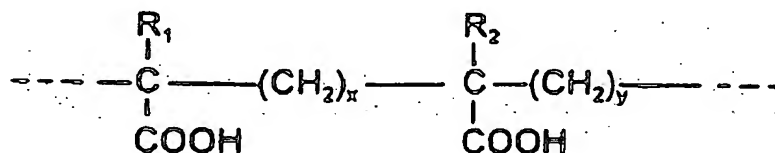
The subject of the present invention is therefore a recombinant live vaccine comprising a viral vector incorporating and expressing in vivo a heterologous nucleotide sequence, preferably a gene for
20 a pathogenic agent, and at least one adjuvant compound chosen from the polymers of acrylic or methacrylic acid and the copolymers of maleic anhydride and alkenyl derivative.

The preferred compounds are the polymers of
25 acrylic or methacrylic acid which are cross-linked, especially with polyalkenyl ethers of sugars or polyalcohols. These compounds are known by the term carbomer (Pharmeuropa Vol. 8, No. 2, June 1996). Persons skilled in the art can also refer to
30 US-A-2.909 462 (incorporated by reference) which describes such acrylic polymers cross-linked with a polyhydroxylated compound having at least 3 hydroxyl groups, preferably not more than 8, the hydrogen atoms of at least three hydroxyls being replaced by
35 unsaturated aliphatic radicals having at least 2 carbon atoms. The preferred radicals are those containing from 2 to 4 carbon atoms, e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated radicals may themselves contain other substituents,

such as methyl. The products sold under the name Carbopol® (BF Goodrich, Ohio, USA) are particularly appropriate. They are cross-linked with an allyl sucrose or with allyl pentaerythritol. Among them,
5 there may be mentioned Carbopol® 974P, 934P and 971P.

Among the copolymers of maleic anhydride and alkenyl derivative, the copolymers EMA® (Monsanto) which are copolymers of maleic anhydride and ethylene, linear or cross-linked, for example cross-linked with
10 divinyl ether, are preferred. Reference may be made to J. Fields et al., Nature, 186: 778-780, 4 June 1960, incorporated by reference.

From the point of view of their structure, the polymers of acrylic or methacrylic acid and the
15 copolymers EMA® are preferably formed of basic units of the following formula:



in which:

- 20 - R₁ and R₂, which are identical or different, represent H or CH₃
- x = 0 or 1, preferably x = 1
- y = 1 or 2, with x + y = 2

For the copolymers EMA®, x = 0 and y = 2. For
25 the carbomers, x = y = 1.

The dissolution of these polymers in water leads to an acid solution which will be neutralized, preferably to physiological pH, in order to give the adjuvant solution into which the vaccine itself will be
30 incorporated. The carboxyl groups of the polymer are then partly in COO⁻ form.

Preferably, a solution of adjuvant according to the invention, especially of carbomer, is prepared in distilled water, preferably in the presence of sodium
35 chloride, the solution obtained being at acidic pH. This stock solution is diluted by adding it to the

required quantity (for obtaining the desired final concentration), or a substantial part thereof, of water charged with NaCl, preferably physiological saline (NaCl 9 g/l), all at once or in several portions with
5 concomitant or subsequent neutralization (pH 7.3 to 7.4), preferably with NaOH. This solution at physiological pH will be used as it is to reconstitute the vaccine, especially stored in freeze-dried form.

The polymer concentration in the final vaccine
10 composition will be 0.01% to 2% w/v, more particularly 0.06 to 1% w/v, preferably 0.1 to 0.6% w/v.

The invention proves particularly useful for vaccination against animal herpesviruses. The invention relates most particularly to the equine herpesvirus
15 (EHV-1 and EHV-4 in particular), feline herpesvirus (FHV), canine herpesvirus (CHV), avian herpesvirus (Marek and ILTV), bovine herpesvirus (BHV) and porcine herpesvirus (PRV = Aujeszky's disease virus or pseudorabies virus).

20 The subject of the invention is therefore recombinant live vaccines comprising at least one viral vector incorporating and expressing at least one gene of such a herpesvirus and at least one adjuvant in accordance with the invention.

25 By way of example, persons skilled in the art may refer to WO-A-92 15672 (incorporated by reference), which describes the production of expression vectors based on poxviruses capable of expressing such genes. There will be found for example a canarypox expressing
30 the gB, gC and gD genes of EHV-1 (vCP132), which is also applicable to EHV-4, a vaccinia virus expressing these same genes (vP 1043), a vaccinia virus expressing the gI(gB), gIII(gC) and gIV(gD) genes of BHV-1, a canarypox expressing gD of FHV-1, or alternatively
35 recombinants expressing the gII (gB), gIII (gC) and gp50 (gD) of PRV. They can also refer to WO-A-95/26 751 (incorporated by reference), which describes recombinant viruses vCP320, vCP322 and vCP294 expressing the gB, gC and gD genes, respectively, of

CHV. They can also refer to the recombinants expressing FHV, PRV and BHV genes in FR-A-2 647 808, or WO-A-9012882, incorporated by reference.

5 The invention also proves particularly advantageous for vaccination against influenza viruses, as demonstrated here for EIV (equine influenza virus). There may also be mentioned avian influenza (AIV), porcine influenza (swine influenza virus).

10 By way of example, persons skilled in the art can refer to the recombinant canarypox expressing the HA gene of EIV in WO-A-92 15 672.

15 The subject of the invention is therefore recombinant live vaccines comprising at least one viral vector incorporating and expressing at least one gene of such an influenza virus, and at least one adjuvant in accordance with the invention. In particular, the vaccine comprises a mixture of two or three vectors, each incorporating and expressing an HA gene, the genes being obtained from different strains, for example
20 equine influenza strains Prague, Kentucky and Newmarket. Similarly, a single vector may be used to incorporate and express the HAs of 2 or of the 3 strains.

25 The invention also applies to other animal pathogens, such as in particular FeLV (see also canarypox recombinants in WO-A-92 15672 by way of example, expressing env, gag = vCP93 and vCP97), tetanus (see also WO-A-92 15672 and the recombinants vCP161 and vP1075, canarypox and vaccinia, expressing
30 the tetanus toxin), Carré's disease virus (canine distemper virus or CDV) (see recombinant vCP 258 in WO-A-95 27780, incorporated by reference).

35 The subject of the invention is therefore recombinant live vaccines comprising at least one viral vector incorporating and expressing at least one gene of such a virus.

The subject of the invention is also multivalent recombinant vaccines, that is to say containing two or more recombinant vectors expressing

antigens of two or more diseases, in the form of a mixture in an adjuvant solution in accordance with the invention.

Moreover, the invention applies to the use of
5 any type of viral expression vector, such as poxvirus (vaccinia virus, including NYVAC according to WO-A-92/15672, fowlpox, canarypox, pigeonpox, swinepox and the like), adenovirus, herpesvirus. Canarypox, e.g. ALVAC (WO-A-95/27780 and WO-A-92/15672) is found to be
10 particularly appropriate in the context of the present invention.

In a ready-for-use, especially reconstituted, vaccine, the viral vector is present in the quantities normally used and described in the literature.

15 The recombinant live vaccines generally exist in a freeze-dried form allowing their storage and are reconstituted immediately before use in a solvent or excipient, which will be here the solution of adjuvant in accordance with the invention.

20 The subject of the invention is therefore also a vaccination set comprising, packaged separately, freeze-dried vaccine and a solution of the adjuvant compound according to the invention for the reconstitution of the freeze-dried vaccine.

25 The subject of the invention is also a method of vaccination consisting in administering, by the parenteral, preferably subcutaneous, intramuscular or intradermal, route or by the mucosal route a vaccine in accordance with the invention at the rate of one or
30 more administrations, optionally with a preliminary step of reconstituting the freeze-dried vaccine (the recombinant vector) in a solution of adjuvant compound.

One objective of such a method may be to protect animals from the clinical point of view and to
35 reduce viral excretion, which corresponds in particular to the case of herpesviruses.

Another objective may be to increase the immune response and to make it occur earlier, especially by

inducing antibodies starting from the first administration.

The subject of the invention is also the use of the adjuvant compounds in accordance with the invention for the production of recombinant live vaccines, especially conferring an improved and earlier immune response and/or an increased reduction in viral excretion. Reference may be made to what was said above.

The invention will now be described in greater detail with the aid of the embodiments taken by way of nonlimiting examples and referring to the drawing in which:

- Figure 1 represents the nucleotide sequence of the EIV HA gene of the EIV Newmarket 2/93 strain;

- Figure 2 represents the nucleotide sequence of the FHV-1 gC gene;

- Figure 3 represents a graph showing the variation of viral excretion after experimental infection in horses vaccinated with the aid of different vaccines against the equine herpesvirus.

EXAMPLES

Example 1: Generation of the donor plasmids for the sites of insertion C3, C5 and C6 into the canarypox virus "ALVAC"

The donor plasmids for the different sites of insertion into the canarypox virus "ALVAC" (Tartaglia et al. Virology, 1992. 188. 217-232, incorporated by reference) are described in Application WO-A-95/27780, Example 20.

These plasmids were designated in this application in the following manner:

"plasmid VQH6CP3LSA.2" for the "C3" site

"plasmid HC5LSP28" for the "C5" site

"plasmid pC6L" for the "C6" site.

Example 2: Generation of the recombinant virus vCP258 (ALVAC/CDV HA+F)

The Onderstepoort strain of the CDV virus was used to isolate the HA and F genes (sequence of the HA

gene described by Curran et al. Virology. 1991. 72. 443-447, and sequence of the F gene described by Barrett et al. Virus Research. 1987. 8. 373-386, both incorporated by reference).

5 The construction of the donor plasmid pMM103 for the insertion of the expression cassettes H6 vaccinia promoter-CDV F gene and H6 vaccinia promoter-CDV HA gene into the C6 locus of the ALVAC virus is described in Example 19 of Application WO-A-95/27780.

10 This plasmid was used as donor plasmid for in vitro recombination (Piccini et al. Methods in Enzymology. 1987. 153. 545-563, incorporated by reference), with the ALVAC virus to generate the recombinant virus designated vCP258 as in Example 19 of
15 the abovementioned application.

Example 3: Generation of the recombinant virus vCP1502 (ALVAC/EIV HA Prague)

The sequence of the HA gene (EIV Prague strain) is presented in Figure No. 23 of Application
20 WO-A-92/15672. The viral RNA of the genome of the equine influenza virus strain Prague 56 was extracted from 100 µl of a viral suspension of this virus with the "Total RNA Separator kit" extraction kit from CLONTECH (Palo Alto, CA) (Cat#K1042-1). The RNA pellet
25 was taken up in 10 µl of ultrapure water and a complementary DNA synthesis reaction, followed by a PCR reaction (= "RT-PCR" reaction) was carried out taking as template 2 µl of purified viral RNA and the following oligonucleotides:

30 TAY51A (SEQ ID No.1) (70 mer)

5'CGCGGCCATCGCGATATCCGTTAAGTTTGTATCGTAATGAACACTCAAATTCT
AATATTAGCCACTTCGG3'

and TAY53A (SEQ ID No.2) (36 mer)

5'CGCGCGGCGGTACCTTATATACAAATAGTGCACCGC3'

35 in order to amplify the Prague EIV HA gene. The PCR fragment thus obtained was ligated into the vector pCRII (Invitrogen, San Diego, CA) to give the plasmid pJT007.

The plasmid pJT007 was digested with NruI and Asp718 in order to isolate an NruI-718 fragment of about 1800 bp containing the end of the H6 promoter and the Prague 56 HA gene in its entirety. This fragment
5 was ligated with the donor plasmid C5 HC5LSP28, previously digested with NruI and Asp718, to finally give the plasmid pJT008. This plasmid contains the expression cassette H6-Prague 56 HA gene in the C5 locus of the ALVAC virus. The structure of this plasmid
10 was verified by sequencing and complete restriction map.

This plasmid is the donor plasmid for the insertion of the expression cassette H6-Prague 56 HA gene into the C5 locus.

15 After linearizing with NotI, the plasmid pJT008 was used as donor plasmid for *in vitro* recombination (Piccini et al. Methods in Enzymology. 1987. 153. 545-563) with the ALVAC virus in order to generate the recombinant virus designated vCP1502.

20 **Example 4: Generation of the recombinant virus vCP1529 (ALVAC/EIV HA Kentucky 1/94)**

The viral RNA of the genome of the equine influenza virus strain Kentucky 1/94 (Daly et al. J. Gen. Virol. 1996. 77. 661-671, incorporated by
25 reference) was extracted with 100 µl of a viral suspension of this virus with the "Total RNA Separator kit" extraction kit from CLONTECH (Palo Alto, CA) (Cat#K1042-1). The RNA pellet was taken up in 10 µl of ultrapure water and RT-PCR reaction was carried out
30 taking as template 2 µl of purified viral RNA and the following oligonucleotides:

TAY55A (SEQ ID No.3) (70 mer)

5'CGCGGCCATCGCGATATCCGTTAAGTTTGTATCGTAATGAAGACAACCATTAT
TTTGATACTACTGACCC3'

35 and TAY57A (SEQ ID No.4) (42 mer)

5'CGCGCGGCGGTACCTCAAATGCAAATGTTGCATCTGATGTTG3'

in order to amplify the HA gene. The PCR fragment thus obtained was ligated into the vector pCRII (Invitrogen, San Diego, CA) to give the plasmid pJT001. The sequence

of the Kentucky 1/94 strain EIV HA gene cloned into the plasmid pJT001 is not different from the sequence of the Kentucky 1/94 strain EIV HA gene available in the GenBank databank (accession number L39914, incorporated by reference).

The plasmid pJT001 containing the HA gene (Kentucky 1/94) was digested with NruI and Asp718 in order to isolate an NruI-Asp718 fragment of 1800 bp (containing the end of the H6 promoter and the Kentucky 1/94 HA gene in its entirety). This fragment was ligated with the donor plasmid C5 HC5LSP28, previously digested with NruI and Asp718, to finally give the plasmid pJT005. This plasmid contains the expression cassette H6-Kentucky 1/94 HA gene in the C5 locus of the ALVAC virus. The structure of this plasmid was verified by sequencing and complete restriction map.

This plasmid is a donor plasmid for the insertion of the expression cassette H6-Kentucky 1/94 HA gene into the C5 locus.

After linearizing with NotI, the plasmid pJT005 was used as donor plasmid for *in vitro* recombination (Piccini et al. Methods in Enzymology. 1987. 153. 545-563) with the ALVAC virus in order to generate the recombinant virus designated vCP1529.

Example 5: Generation of the recombinant virus vCP1533 (ALVAC/EIV HA Newmarket 2/93)

The viral RNA of the genome of the equine influenza virus strain Newmarket 2/93 (Daly et al. J. Gen. Virol. 1996. 77. 661-671) was extracted from 100 µl of a viral suspension of this virus with the "Total RNA Separator kit" extraction kit from CLONTECH (Cat#K1042-1). The RNA pellet was taken up in 10 µl of ultrapure water and an RT-PCR reaction was carried out taking as template 2 µl of purified viral RNA and the following oligonucleotides:

CCL007 (SEQ ID No.5) (40 mer)

5'TTGTCGACTCAATCATGAAGACAACCATTTGATACT3'

and CCL0018 (SEQ ID No.6) (34 mer)

5'TTGGATCCTTACTCAAATGCAAATGTTGCAYCTG3'

in order to amplify the HA gene. The PCR fragment thus obtained was ligated to the vector pCRII (Invitrogen, San Diego, CA) to give the plasmid pCCL026. The sequence of the HA gene (EIV Newmarket 2/93 strain) is presented in Figure No. 1 (SEQ ID No.7).

The plasmid pCCL026 containing the HA gene (Newmarket 2/93 strain) was digested with SpeI and AccI. The following oligonucleotides:

TAY99N (SEQ ID No.8) (74 mer)

5'CTAGTTCGCGATATCCGTTAAGTTTGTATCGTAATGAAGACAACCATTATTTT
GATACTACTGACCCATTGGGT3'

and TAY100N (SEQ ID No.9) (72 mer)

5'AGACCCAATGGGTCAGTAGTATCAAATAATGGTTGTCTTCATTACGATACAA
ACTTAACGGATATCGCGAA3'

were annealed and ligated with the plasmid pCCL026 digested with SpeI+AccI to give the plasmid pJT003. The double-stranded oligonucleotide TAY99N/TAY100N contains the 3' region of the H6 promoter up to the NruI site and the first 40 coding bases of the HA gene.

The plasmid JT003 was digested with NruI and XhoI in order to isolate an NruI-XhoI fragment of about 1800 bp containing the end of the H6 promoter and the HA gene in its entirety. This fragment was ligated with the donor plasmid C5 HC5LSP28, previously digested with NruI and XhoI, to finally give the plasmid pJT004. This plasmid contains the expression cassette H6-Newmarket 2/93 HA gene in the C5 locus of the ALVAC virus. The structure of this plasmid was verified by sequencing and complete restriction map.

This plasmid is the donor plasmid for the insertion of the expression cassette H6-Newmarket 2/93 HA gene into the C5 locus.

After linearizing with PvuI, the plasmid pJT004 was used as donor plasmid for the in vitro recombination (Puccini et al. Methods in Enzymology. 1987. 153. 545-563) with the ALVAC virus in order to generate the recombinant virus designated vCP1533.

Example 6: Generation of the recombinant virus vCP132 (ALVAC/EHV-1 gB+gC+gD)

The construction of the recombinant virus is described in Examples 25 and 26 of Application WO-A-92/15672. This virus was generated by *in vitro* recombination between the ALVAC virus and the donor
5 plasmid pJCA049. This plasmid contains the following 3 expression cassettes cloned into the site of insertion C3:

- I3L vaccinia promoter-EHV-1 gB gene
- H6 vaccinia promoter-EHV-1 gC gene
- 10 42K entomopox promoter-EHV-1 gD gene

The sequences of the EHV-1 gB, gC and gD genes are described in Application WO-A-92/15672 in Figures No. 2 (sequence of the EHV-1 gene gp13 = gC), No. 6 (sequence of the EHV-1 gene gp14 = gB) and No. 12
15 (sequence of the EHV-1 genes gD, gp63 and gE).

Example 7: Generation of the recombinant virus vCP243 (ALVAC/FHV-1 gB+gC+gD)

The sequence of the FHV-1 gB gene (CO strain) is presented in Figure No. 34 of Application
20 WO-A-90/12882.

The FHV-1 gC gene (CO strain) (sequence presented in Figure No. 2) (SEQ ID No. 10) was cloned from the EcoRI F fragment (7.6 kbp). It has a size of 1599 bp and encodes a protein of 533 amino acids.

25 The FHV-1 gD gene (CO strain) (sequence presented in Figure No. 28 of Application WO-A-92/15672) was cloned from the EcoRI M fragment (4.4 kbp) (plasmid pFHVEcoRIM).

Construction of the expression cassette I3L-FHV-1 gB gene mutated at the level of the signals for
30 early termination of transcription (TTTTTNT).

The following oligonucleotides:

MP287 (SEQ ID No.11) (20 mer)

5'GATTAAACCTAAATAATTGT3'

35 and JCA158 (SEQ ID No.12) (21 mer)

5'TTTTCTAGACTGCAGCCCGGGACATCATGCAGTGGTTAAAC3'

were used for a PCR amplification with the template of a plasmid containing the I3L vaccinia promoter (Rivière et al. J. Virol. 1992. 66. 3424-3434, incorporated by

reference). in order to generate a blunt-ended XbaI fragment of 120 bp (containing the I3L vaccinia promoter) = fragment A. The following oligonucleotides: JCA213 (SEQ ID No.13) (18 mer)

5 5'GGGTTTCAGAGGCAGTTC3'

and JCA238 (SEQ ID No.14) (21 mer)

5'ATGTCCACTCGTGGCGATCTT3'

were used to generate, by PCR from the template of the plasmid pJCA001, a blunt-ended BamHI fragment of 720 bp (containing the 5' part of the FHV-1 gB gene) = fragment B.

Fragment A was digested with XbaI, and then phosphorylated. Fragment B was digested with BamHI, and then phosphorylated. Fragments A and B were then ligated together with the vector pBluescript SK+, previously digested with XbaI and BamHI, to give the plasmid pJCA075.

The following oligonucleotides:

JCA158 (SEQ ID No.15) and JCA211 (SEQ ID No.16) (21 mer):

5'GTGGACACATATAGAAAGTCG3'

were used to generate, by PCR from the template of the plasmid pJCA075, a blunt-ended XbaI fragment of 510 bp (containing the I3L promoter fused to the 5' part of the FHV-1 gB gene mutated at the level of the signal TTTTNT) = fragment C.

The following oligonucleotides:

JCA212 (SEQ ID No.17) 21 mer)

5'CACCTTCAGGATCTACTGTCG3'

30 and JCA213 (SEQ ID No.13) (18 mer)

were used to generate, by PCR from the template of the plasmid pJCA001, a blunt-ended BamHI fragment of 330 bp (containing the central part of the FHV-1 gB gene) = fragment D.

35 Fragment C was digested with XbaI, and then phosphorylated. Fragment D was digested with BamHI, and then phosphorylated. Fragments C and D were then ligated together with the vector pBluescript SK+,

previously digested with XbaI and BamHI to give the plasmid pJCA076.

The following oligonucleotides:

JCA239 (SEQ ID No.18) (24 mer)

5 'ACGCATGATGACAAGATTATTATC3'

and JCA249 (SEQ ID No.19) (18 mer)

5'CTGTGGAATTCGCAATGC3'

were used to generate, by PCR from the template of the plasmid pJCA001, a blunt-ended EcoRI fragment of 695 bp (containing the first 3' part of the FHV-1 gB gene) = fragment E. The following oligonucleotides:

JCA221 (SEQ ID No.20) (48 mer)

5'AAACTGCAGCCCGGGAAGCTTACAAAAATTAGATTTGTTTCAGTATC3'

and JCA247 (SEQ ID No.21) (36 mer)

15 5'GGTATGGCAAATTTCTTTCAGGGACTCGGGGATGTG3'

were used to generate, by PCR from the template of the plasmid pJCA001, a blunt-ended PstI fragment of 560 bp (containing the second 3' part of the FHV-1 gB gene mutated at the level of the signal TTTTNT) = fragment F.

Fragment E was digested with EcoRI, and then phosphorylated. Fragment F was digested with PstI, and then phosphorylated. Fragments E and F were then ligated together with the vector pIBI24 (International Biotechnologies Inc., New Haven, CT), previously digested with EcoRI and PstI, to give the plasmid pJCA077 (containing the cassette I3L vaccinia promoter FHV-1 B gene).

Construction of the expression cassette 42K-FHV-1 gD

The following oligonucleotides:

RG286 (SEQ ID No.22) (17 mer)

5'TTTATATTGTAATTATA3'

and M13F (SEQ ID No.23) (17 mer)

35 5'GTAAAACGACGGCCAGT3'

were used to generate, by PCR from the template of the plasmid containing the 42K Entomopoxvirus AmEPV promoter (described in Example 21 of Patent US-A-5,505,941), a blunt-ended EcoRI fragment of 130 bp

(containing the 42K entomopox promoter) = fragment A.
The following oligonucleotides:

JCA234 (SEQ ID No.24) (21 mer)

5'ATGATGACACGTCTACATTTT3'

5 and JCA235 (SEQ ID No.25) (21 mer)

5'TGTTACATAACGTACTTCAGC3'

were used to generate by PCR from the template of the
plasmid pFHVEcoRIM, a blunt-ended BamHI fragment of
185 bp (containing the 5' part of the FHV-1 gD gene) =
10 fragment B. Fragment A was digested with EcoRI, and
then phosphorylated. Fragment B was digested with
BamHI, and then phosphorylated. Fragments A and B were
then ligated together with the vector pBluescript SK+,
previously digested with EcoRI and BamHI, to give the
15 plasmid pJCA078.

The plasmid pFHVEcoRIM (see above) was digested
with BamHI and XhoI in order to isolate the BamHI-XhoI
fragment of 1270 bp (containing the 3' part of the
FHV-1 gD gene). This fragment was then ligated with the
20 vector pIBI24, previously digested with BamHI and XhoI,
in order to give the plasmid pJCA072. The following
oligonucleotides:

JCA242 (SEQ ID No.26) (18 mer)

5'GAGGATTTCGAAACGGTCC3'

25 and JCA237 (SEQ ID No.27) (53 mer)

5'AATTTTCTCGAGAAGCTTGTTAACAAAAATCATTAAGGATGGTAGATTGCATG3'

were used to generate, by PCR from the pFHVEcoRIM
template, an XbaI-XhoI fragment of 290 bp. This
fragment was digested with XbaI and XhoI = fragment C
30 (containing the end of the FHV-1 gD gene).

The plasmid pJCA072 was digested with XbaI and
XhoI in order to isolate the XbaI-XhoI fragment of
3575 bp (vector pIBI24 + start of the 3' part of the
FHV-1 gD gene) = fragment D. Fragments C and D were
35 then ligated together in order to give the plasmid
pJCA073.

The plasmid pJCA073 was digested with BamHI and
XhoI in order to isolate the BamHI-XhoI fragment of
960 bp (containing the 3' part of the FHV-1 gD gene) =

fragment A. The plasmid pJCA078 was digested with HpaI and BamHI in order to isolate the HpaI-BamHI fragment of 310 bp (containing the 42K promoter fused to the 5' part of the FHV-1 gD gene) = fragment B. Fragments A and B were ligated together with the vector pBluescript SK+, previously digested with EcoRV and XhoI, in order to give the plasmid pJCA080 (containing the cassette 42K promoter-FHV-1 gD gene).

Construction of the cassette H6-FHV-1 gC

The genomic DNA of the FHV-1 virus (CO strain) was digested with EcoRI and the EcoRI F fragment of about 7500 bp was cloned into pBluescript SK+ to give the plasmid pFHVEcoRIF. The following oligonucleotides:

JCA274 (SEQ ID No.28) (55 mer)

5'CATTATCGCGATATCCGTTAAGTTTGTATCGTAATGAGACGATATAGGATGGGAC3'

and JCA275 (SEQ ID No.29) (18 mer)

5'ACTATTTTCAATACTGAC3'

were used to generate, by PCR from the pFHVEcoRIF template, a fragment which was digested with NruI and SalI in order to give an NruI-SalI fragment of 107 bp (containing the 3' part of the H6 vaccinia promoter fused to the 5' part of the FHV-1 gC gene) = fragment A.

The following oligonucleotides:

JCA276 (SEQ ID No.30) (18 mer)

5'AAATGTGTACCACGGGAC3'

and JCA277 (SEQ ID No.31) (54 mer)

5'AAGAAGCTTCTGCAGAATTCGTTAACAAAAATCATTATAATCGCCGGGGATGAG3'

were used to generate, by PCR from the pFHVEcoRIF template, a fragment which was digested with EcoRV and HindIII in order to give an EcoRV-HindIII fragment of 370 bp (containing the 3' part of the FHV-1 gC gene and the HpaI-EcoRI-PstI-HindIII sites) = fragment B.

The plasmid pJCA020 (see above) was digested with NruI and HindIII in order to isolate the HindIII-NruI fragment (containing the 5' part of the H6 vaccinia promoter) = fragment C. The plasmid pFHVEcoRIF

was digested with BamHI and EcoRV in order to isolate the BamHI-EcoRV fragment of 580 bp (containing the central part of the FHV-1 gC gene) = fragment D. Fragments A and C were ligated together with the vector pBluescript SK+, previously digested with HindIII and SalI in order to give the plasmid pJCA097. Fragments B and D were ligated together with the vector pBluescript SK+, previously digested with BamHI and HindIII, to give the plasmid pJCA099.

10 The plasmid pJCA097 was digested with PstI and SalI in order to isolate the PstI-SalI fragment of 200 bp (containing the cassette H6-5' part of gC) = fragment E. The plasmid pFHV1EcoRIF was digested with BamHI and SalI in order to isolate the SalI-BamHI
15 fragment of 600 bp (2nd central part of FHV-1 gC) = fragment F. Fragments E and F were then ligated together with the vector pBluescript SK+, previously digested with BamHI and PstI, in order to give the plasmid pJCA098. The plasmid pJCA098 was then digested
20 with EcoRI and BamHI in order to isolate the EcoRI-BamHI fragment of 820 bp (containing the cassette H6-5' part of FHV-1gC) = fragment G. The plasmid pJCA099 (see above) was digested with BamHI and HindIII in order to isolate the BamHI-HindIII fragment of 960 bp
25 (containing the 3' part of the FHV-1 gC gene) = fragment H. Fragments G and H were then ligated together with the vector pBluescript SK+, previously digested with EcoRV and HindIII, in order to give the plasmid pJCA100 (containing the expression cassette H6 vaccinia promoter-FHV-1 gC gene).

30 The plasmid pJCA100 was digested with NruI and EcoRI in order to isolate the NruI-EcoRI fragment of 1650 bp containing the 3' part of the H6 promoter fused with the FHV-1 gC gene. This fragment was ligated with
35 the plasmid pJCA053 (cassette VQH6-IBV M in the vector pBluescript SK+), previously digested with NruI and EcoRI, in order to give the plasmid pJCA108 (containing the cassette VQH6-gC in pBluescript SK+). The plasmid pJCA079 (see above) was digested with SmaI and BamHI in

order to isolate the BamHI-SmaI fragment of 840 bp (containing the cassette I3L-5' part of the FHV-1 gB gene) = fragment A. The plasmid pJCA079 was also digested with BamHI and HindIII in order to isolate the
5 BamHI-HindIII fragment of 2155 bp (containing the 3' part of the FHV-1 gB gene) = fragment B. The plasmid pJCA108 (see above) was digested with HindIII and EcoRI in order to isolate the HindIII-EcoRI fragment of 1830 bp (containing the cassette VQH6-FHV-1 gC) =
10 fragment C. The plasmid pJCA080 (see above) was digested with EcoRI and XhoI in order to isolate the EcoRI-XhoI fragment of 1275 bp (containing the cassette 42K-FHV-1 gD gene) = fragment D. Fragments A, B, C and D were then ligated together with the donor plasmid
15 pC6L in order to give the plasmid pJCA109.

This plasmid contains the expression cassettes H6-FHV-1 gene gC, I3L-FHV-1 gB gene and 42K-FHV-1 gD gene in the C6 locus of the ALVAC virus. The structure of this plasmid was verified by sequencing and complete
20 restriction map.

This plasmid is the donor plasmid for the insertion of the expression cassettes H6-FHV-1 gC gene, I3L-FHV-1 gB gene and 42K-FHV-1 gD gene in the C6 locus of the ALVAC virus.

25 After linearizing with NotI, the plasmid pJCA109 was used as donor plasmid for *in vitro* recombination (Piccini et al. Methods in Enzymology. 1987. 153. 545-563) with the ALVAC virus in order to generate the recombinant virus designated vCP243.

30 **Example 8: Adjuvant**

The carbomer used in the vaccines in accordance with the present invention is Carbopol® 974P manufactured by the company BF Goodrich (MW about 3 million).

35 A stock solution containing 1.5% w/v of Carbopol® 974P was first prepared in distilled water containing sodium chloride at 1 g/l.

This stock solution is then used for the manufacture of a solution of Carbopol® in physiological

saline at 4 mg/ml. The stock solution is poured into the entire physiological saline (or optionally into most of it) all at once or optionally in several portions with, each time, adjustment of the pH with the aid of NaOH (for example 1 N or more concentrated) to a value of about 7.3 to 7.4.

A ready-for-use solution of Carbopol® is thereby obtained which can be used by the final user to reconstitute a freeze-dried recombinant vaccine.

Example 9: Vaccination of horses with the aid of the recombinant canarypox vector vCP132 (see Example 6) expressing the glycoproteins gB, gC and gD of the type I equine herpesvirus (EHV-1).

1. Protocol for immunization and challenge:

20 ponies (Welsh mountain ponies) exhibiting no serological signs indicating a recent exposure to EHV-1 and EHV-4 were randomly distributed into 4 groups (A to D) of 5 ponies.

Groups A and B were vaccinated with the recombinant canarypox vCP132 expressing the glycoproteins gB, gC and gD of the Kentucky D strain of EHV-1. The vaccine was reconstituted in sterile water (group A) or in a solution of carbomer 4 mg/ml (group B) according to Example 8.

Group C was vaccinated with a commercial inactivated whole EHV vaccine containing, in a dose volume of 1.5 ml, inactivated EHV-1 and EHV-4 valencies and 6 mg of carbomer.

Group D is the control group in which the animals were vaccinated with a recombinant canarypox virus vCP1502 expressing the HA glycoprotein of the Influenza A/equi-1/Prague56 virus (see Example 3) reconstituted in carbomer under the same conditions as for group B.

The vaccines are described in detail in Table 1:

Groups	Vaccines	Antigens	Diluent/ adjuvant	Dose (1 ml)
A	vCP132	EHV-1	Sterile water	$10^{8.0}$ TCID ₅₀
B	vCP132	EHV-1	Carbopol® 974P	$10^{8.0}$ TCID ₅₀
C	Commercial vaccine	EHV-1 EHV-4	Carbopol®	$10^{7.3}$ TCID ₅₀ before inactivation EHV-1 $10^{7.3}$ TCID ₅₀ before inactivation EHV-4
D	vCP1502	HA-Prague 56	Carbopol® 974P	10^8 TCID ₅₀

Each animal received 1 dose of vaccine corresponding to D0 and D35 by deep intramuscular injection into the neck.

5 On D56, the ponies were challenged by intranasal instillation of 10^5 TCID₅₀ of the Ab4/8 strain of EHV-1.

2. Serological tests

10 Neutralization tests SN were carried out according to the technique described in Thompson et al., Equine Vet. J., 8, 58-65, 1976. The EHV-1 virus (RACH) was used as antigen.

The SN titres are expressed as the reciprocal of the serum dilution giving 50% neutralization (\log_{10}).

3. Virological monitoring:

15 The expression of the virus was monitored daily over 10 days using nasopharyngeal swabs which were collected in virus transporting medium. The swab extracts were titrated on rabbit kidney cells RK13 in
20 microtitre plates. The titres were calculated using the Karber formula expressed in \log_{10} TCID₅₀ per 1 ml.

4. Results:

No significant local or systemic reaction was noted following these vaccinations.

The seroneutralization SN antibody mean responses (log 10 of the dilution causing 50% neutralization) are:

Group	Titre on D.0	Titre on D.56 (before challenge)
A	1.69 ± 0.49	1.93 ± 0.15
B	1.69 ± 0.47	2.61 ± 0.42
C	1.19 ± 0.30	2.47 ± 0.32
D	1.57 ± 0.45	1.55 ± 0.37

A significant increase in the antibody titre is observed with the vaccine vCP132 in carbomer.

All the 5 control ponies excrete the virus through the nasopharynx. The viral excretion in these nonvaccinated ponies continued for an average of 5 days, with a maximum viral excretion at 4 days post-infection.

All the ponies in groups A, C and D excrete a virus after challenge. By contrast only two ponies out of the 5 ponies in group B vaccinated according to the invention excrete the virus. In addition, the quantity of virus excreted in group B is significantly less than the quantity excreted by the other groups including group C. Likewise, the duration of excretion in the animals in group B is much shorter than in the other groups.

Reference may be made to Figure 1 and to the area under the curve values given below, which show very clearly the virtual absence of viral excretion in the ponies vaccinated with vCP132 in the presence of carbomer. The result is very significant if it is compared in particular with the commercial vaccine. A significant reduction in viral excretion is observed in the animals in group B compared with the controls, whereas no significant difference is observed between

the animals in group C and the controls. This reduction by a remarkable and unexpected level in the excretion of virus is particularly advantageous because of its very favourable indications on the limitation of the transmission of the virus from horse to horse.

Total virus per pony (area under the curve):

A: 17.1
B: 3.0
C: 9.7
10 D: 16.3

Example 10: Vaccination of horses with the aid of the canarypox vector vCP1533 (see Example 5) recombinant expressing the HA glycoprotein of the Influenza A/equi-2/Newmarket/2/93 virus in the presence of carbomer:

1. Protocol for immunization and challenge:

20 ponies (Welsh mountain ponies), 7 to 8 months old having no detectable antibodies against the H3N8 and H7N7 viruses, measured by the SRH (for single radial haemolysis) test were used in this study. The negative status of the animals makes it possible to study, under the best conditions, the efficacy of the various vaccines in terms of humoral response. The ponies were randomly distributed into 4 groups (A to D) of 5 to 6 ponies.

The ponies in group A were vaccinated with the aid of a recombinant canarypox (vCP1533) expressing the HA glycoprotein of the influenza A/equi-2/Newmarket/2/93 virus. This vaccine was reconstituted in a solution containing 4 mg/ml of carbomer, Carbopol® 974P.

Group B was vaccinated with a commercial vaccine containing, in a dose volume of 1.5 ml, a mixture of 3 inactivated strains of influenza, namely Prague/56, Suffolk/89 and Miami/63, tetanus toxoid, as well as carbomer (4 mg) and aluminium hydroxide (2.2 mg) as adjuvants.

Group C was vaccinated with the aid of a vaccine C comprising 2 influenza inactivated valencies,

namely Prague/56, Newmarket/2/93 as well as tetanus toxoid in aluminium hydroxide.

Group D was vaccinated with the aid of a recombinant canarypox vector vCP132 seen above and reconstituted with a solution containing 4 mg/ml of carbomer 974P. The latter group served as control for the challenge.

The vaccines are described in detail in Table II:

10

Groups	Vaccines	Antigens	Diluent/ adjuvant	Dose (1 ml)
A	vCP1533	HA- Newmarket/2/93	Carbopol® 974P	$10^{7.7}$ TCID ₅₀
B	Commercial vaccine	Prague/56; Suffolk/89 Miami/63; tetanus toxoid	Carbopol® Al(OH) ₃	15 µg HA of each strain
C	Vaccine C	Prague/56; Newmarket/2/93 tetanus toxoid	Al(OH) ₃	15 µg HA of each strain
D	vCP132	gB, gC, gD- EHV-1	Carbopol® 974P	$10^{8.0}$ TCID ₅₀

2 doses of 1 ml of each vaccine were administered to each animal at an interval of 5 weeks by deep intramuscular injection into the neck.

15

2 weeks after the second vaccination, each pony was infected by exposure to an aerosol obtained from about 20 ml of allantoic fluid for a total of $10^{7.3}$ EID₅₀ of influenza A-equi-2-/Sussex/89 virus, using an ULTRA 2000 model spraying device (De Villbiss, Somerset PA) as described by Mumford et al, Equine Vet, J., 22: 93-98, 1990.

20

2. Serological test:

Samples of whole blood were collected on the following days: 0 (the same day as and before the first vaccination), 7, 14, 35 (the same day as and before the

25

second vaccination), 49 (the same day as and before the challenge), 56 and 63.

5 The serum was prepared and stored and preserved by freezing at -20°C until it is used. All the sera were tested for the presence of SRH antibody against Influenza A/equi-1/Prague/56 and Influenza A/equi-2/Newmarket/2/93 as described by Wood et al. (J. Hyg., 90: 371-384, 1983).

10 The diameters of the haemolysis zones were measured in two directions at right angles using an automated reader. The surface area of the zones was calculated and an increase of 50% was considered as being significant. The titres were expressed in mm^2 of haemolysis.

15 3. Virological monitoring.

Viral excretion was monitored daily over 10 days by collecting naso pharyngeal swabs in a virus transporting medium. The exudate from each swab was diluted by 10-fold serial dilutions in PBS at pH 7.2 and 0.1 ml of each dilution was inoculated into the allantoic space of 10 day-old embryonated eggs. The viral titre ($\text{EID}_{50}/\text{ml}$) in the swab extracts was calculated from the haemagglutinating activity in the allantoic fluids collected after incubating the eggs at 25 34°C for 72 hours.

4. Results.

No significant local or systemic reaction was observed following the first vaccination with the exception of one horse in group B.

30 It should be noted that the strains Suffolk and Newmarket are similar (Daly et al., J. Gen. Virol. 1996, 661-671) which makes comparison with the commercial vaccine perfectly valid under the trial conditions.

35 None of the ponies had a detectable SRH antibody against Influenza A/equi-2/Newmarket/2/93 or Influenza A/equi-1/Prague/56 at the beginning of the study. The serological results 1 week after the first vaccination showed that none of the ponies was

previously infected with Influenza (no observable booster effect).

2 weeks after the first vaccination, none of the ponies developed a detectable antibody response against Influenza A/equi-1/Prague/56. In addition, there was no detectable SRH antibody against Influenza A/equi-2/Newmarket/93 in 6 animals out of 6 vaccinated with vaccine C, in 4 animals out of 5 vaccinated with the commercial vaccine B and in the control group D. By contrast, a very high SRH antibody titre was observed in all the 5 ponies vaccinated with canarypox in the presence of the carbomer adjuvant: mean 155.4 ± 32.9 . Table III below presents the results obtained animal per animal as regards the SRH antibody titres.

Table III SRH results (mm²)

Ponies	Group	PRAGUE (D0, D7, D14)	NEWMARKET		
			D0	D7	D14
M26	A	0	0	0	158.0
M27	A	0	0	0	104.2
M28	A	0	0	0	160.3
M29	A	0	0	0	196.4
M30	A	0	0	0	158.0
M31	B	0	0	0	0
M32	B	0	0	0	0
M33	B	0	0	0	0
M34	B	0	0	0	0
M35	B	0	0	0	81.2
M36	C	0	0	0	0
M37	C	0	0	0	trace
M38	C	0	0	0	0
M39	C	0	0	0	0
M40	C	0	0	0	trace
M41	C	0	0	0	0
M42	D	0	0	0	0
M43	D	0	0	0	0
M44	D	0	0	0	0
M45	D	0	0	0	0
M46	D	0	0	0	0

5 The vaccine according to the invention leads to
the appearance of a high antibody titre from 14 days
after the first vaccination whereas, overall, for
vaccines B and C, the first vaccination does not cause
on this date the appearance of antibodies at detectable
levels. Such an early production of such a titre is a
10 remarkable and unexpected result which has never been
observed before.

Example 11: Vaccination of horses with the aid
of the canarypox vector vCP1502 (see Example 3)
recombinant expressing the HA glycoprotein of the

influenza A/equi-1/Prague 56 virus in the presence of carbomer

5 The controls of Example 1, vaccinated with vCP1502, were also monitored from the serological point of view.

Table IV below shows the IHA (inhibition of haemagglutination) titres obtained in the animals immunized with 10^8 pfu of vCP1502 with Carbopol® 974P at 0 (1st injection V1) and 35 (2nd injection V2) days.

10

Table IV

Ponies	Anti-H7N7 IHA titres				
	Day 0 (V1)	Day 7	Day 14	Day 35 (V2)	Day 56
RM16	0	0	128	64	128
RM17	0	0	32	128	256
RM18	0	0	16	64	512
RM19	0	0	32	32	256
RM20	0	0	128	64	128

15 As in the preceding example, it is observed that the injection of a canarypox -EIV (expressing the HA gene of the A equi-1/Prague virus) mixed with carbomer allows high specific IHA titres to be obtained from D14 after a vaccination. Remarkably, these high titres are further significantly increased after a booster, reaching a very high mean titre, of a level
20 which has never been observed before for the HA antigen of EIV H7N7 virus on horses which have not undergone promostimulation.

Example 12: Application in cats

25 The recombinant virus tested is a recombinant canarypox virus expressing the gB, gC and gD genes of the feline herpesvirus (Feline Herpesvirus = FHV). This recombinant virus is identified vCP243 (see Example 7).

30 The protocol for vaccination/challenge in the FHV model is the following.

Group	Number of cats	Vaccine	Diluent/ adjuvant	Dose
A	6	vCP243	water	$10^{7.5}$ pfu
B	6	vCP243	Carbopol® 974P	$10^{7.5}$ pfu
C	6	CORIFELIN®	---	1 commercial dose
D (controls)	6	---	---	---

The cats are vaccinated on D0 and D28 by the subcutaneous route.

5 The vaccine CORIFELIN® is a subunit FHV vaccine marketed by Merial, Lyon, France, comprising at least 200 IDR units of FHV viral fractions, 25 µg of purified feline calicivirus antigen, 0.1 mg of thiomersal and the oily excipient QS 1 ml.

10 The challenge is carried out on D49, by the oronasal route for an FHV challenge strain.

The clinical monitoring is carried out for 14 days after challenge, noting the clinical signs (noting of the clinical signs according to the rules of the European Pharmacopoeia).

15 Protection is assessed after challenge on the following criteria:

- mean clinical scores for each group, compared with each other and with the mean clinical score for the control group

20 - level of FHV viral excretion after challenge (measurement of the viral load in pharyngeal swabs prepared daily from D0 to D10 after challenge)

- FHV virus neutralizing antibody titres on blood samples collected on D0, D28, D49, D63.

25 For all these criteria, the mean levels for each group are also compared with each other and with the mean level for the control group.

Example 13: Application in dogs

The recombinant virus tested is a recombinant canarypox virus expressing the HA and F genes of the Carré's disease virus (Canine Distemper Virus, CDV).
5 This recombinant virus is identified vCP258 (see Example 2).

The protocol for vaccination/challenge in the CDV model is the following.

Group	Number of dogs	Vaccine	Diluent/ adjuvant	Dose
A	6	vCP258	water	$10^{7.0}$ pfu
B	6	vCP258	Carbopol® 974P	$10^{7.0}$ pfu
C	6	EURICAN®	---	1 commercial dose
D (controls)	6	---	---	---

10

The dogs are vaccinated on D0 and D28 by the subcutaneous route.

The vaccine EURICAN® (CHPPI2) is a live vaccine marketed by Merial, Lyon, France. One commercial dose
15 contains a minimum of 10^4 pfu of the CDV Onderstepoort vaccinal strain.

The challenge is made on D56 by intracranial administration of a 1/10 dilution of the CDV "Snyder-Hill" challenge strain (batch prepared and provided by
20 USDA). Clinical monitoring is performed for 21 days after challenge, noting the clinical signs (noting of the clinical signs according to the rules of the European Pharmacopoeia).

Protection is assessed after challenge on the
25 following criteria:

- mean clinical scores for each group, compared with each other and with the mean clinical score of the control group

- CDV viraemia level after challenge (measurement of the viral load in the lymphocytes on D56, D61, D63, D66, D70, D77).
- CDV virus neutralizing antibody titres on D0, D14, 5 D28, D42, D56, D63, D77.

For all these criteria, the mean levels for each group are also compared with each other and with the mean level for the control group.

Example 14: Vaccination of horses with a single dose of the canary pox vectors vCP1533 and vCP1529 recombinants (see Examples 4, 5 and 10) in the presence of carbomer:

1. Protocol for immunization and challenge:

10 Welsh Mountain ponies, 12 to 15 months-old and seronegative to influenza (no detectable antibodies to either Influenza A / equi-2 / Newmarket / 2 / 93 or Influenza A / equi-2 / Newmarket / 1 / 93 by SRH test) were randomly divided into two groups of 5 animals each.

Each animal in the test group was vaccinated on day zero with a single 1 ml dose of a vaccine containing 7.48 log₁₀ FAID₅₀ (fluorescence assay infectious dose 50 percent) of a recombinant canarypox virus containing and expressing the HA glycoprotein of Influenza A / equi-2 / Newmarket / 2 / 93 (VCP1533), 7.53 log₁₀ FAID₅₀ of a recombinant canarypox virus containing and expressing the HA glycoprotein of Influenza A / equi-2 / Kentucky / 94 (VCP1529) and 4 mg of Carbomer 974P per ml. The vCP1529 and vCP1533 recombinants were as described in Examples 4 and 5 herein, respectively. The vaccine was delivered by deep intramuscular injection.

The five animals in the control group remained unvaccinated.

All the ponies are challenged on Day 14 by exposure to an aerosol generated from approximately 20 ml of allantoic fluid containing 6.3 log₁₀ EID₅₀ (Egg Infectious dose 50 percent) per ml of influenza A / equi-2 / Newmarket / 5 / 03 using a model ULTRA NEB 2000 (DeVillbiss, Somerset, PA). Following vaporization, the ponies were held in the challenge box for at least an additional 60 minutes.

2. Serological test

Samples of whole blood were collected on the following days: 0 (the same day as and before the vaccination), 14 (the same day as and before challenge), 21 and 28.

The serum was prepared and stored at -20°C until used. All sera samples were tested for the presence of SRH antibody against Influenza A / equi-2 / Newmarket / 2 / 93 and Influenza A / equi-2 / Newmarket / 1 / 93 as previously described.

The diameters of the haemolysis zones were measured in two directions at right angles using an automated reader. The surface area of the zones was calculated and an increase of 50% or 25 mm² was considered significant. The titres were expressed in mm² of haemolysis.

3. Virological Monitoring

Viral excretion was monitored daily over 10 days by collecting naso swabs in a virus transporting medium as previously described. The exudates from each swab was diluted by 10-fold serial dilutions in PBS at pH 7.2 and 0.1 ml of each dilution was inoculated into the allantoic space of 10 day-old embryonated eggs. The viral titre (EID₅₀/ml) in the swab extracts was calculated from the haemagglutinating activity in the allantoic fluids collected after incubating the eggs at 34°C for 72 hours.

4. Symptom Monitoring

Each pony was examined each of the 14 days following challenge for the occurrence of symptoms associated with equine influenza: fever, lethargy, anorexia, coughs and nasal discharge, as well as general condition.

The presence of anorexia was indicated by a score of 1, absence of anorexia received a score of 0.

Dyspnea was indicated as present by a score of 1, or absent by a score of 0.

Nasal discharge was scored on the basis of intensity, extent and type:

- | | |
|------------|--|
| Intensity: | absent (0);
slight discharge when looking into the nose (1);
moderate discharge readily observed when looking at the nares (2);
copious discharge with large collections of discharge in the nares (3). |
| Extent: | unilateral - U (1);
Bilateral - B (2). |
| Type: | serous discharge - SD (1);
mucopurulent discharge – MPD (2). |

A daily score was calculated as the average of the scores for intensity, extent and type (I+E+T)/3.

Scores for temperature were based on the designation of a temperature over 38.9°C as hyperthermia. Accordingly, a temperature of below 38.9°C received a score of 0, and a temperature equal to or greater than 38.9°C received a score of 1.

The absence of a cough was given a score of 0. The presence of a slight cough, at a frequency of once in 20 minutes, received a score of 1. The presence of a moderate cough, at a frequency of two or more occurrences in 20 minutes, received a score of 2.

The general condition of each animal was also monitored, with a score of 0 for normal, apathy received a score of 1, depressed received a score of 2, and prostrated received a score of 3.

5. Results

The results clearly demonstrated protection against symptoms and viral excretion after a single, 1 ml dose of the vaccine. By day 14, prior to challenge, the animals of the vaccinated group had developed significant antibodies against Influenza A / equi-2 / Newmarket / 2 / 93 and Influenza A / equi-2 / Newmarket / 1 / 93 which continued to rise in the two weeks after challenge. In contrast, at day 21, 7 days after challenge, the control group had significantly lower antibody titres. The antibody titre results are depicted in tables VII and VIII.

Table VII: SRH TEST RESULTS (mm²) ANTIGEN : NEWMARKET/2/93

GROUP	FOAL	Day 0	Day 14	Day 21	Day 28
Vaccinated	M048	0.0	87.9	65.3	117.6
	M049	0.0	119.6	155.7	106
	M050	0.0	115.6	100.4	113.6
	M051	0.0	107.9	149	162.6
	M052	0.0	82.8	136	158
	MEAN	0.0	102.8	121.3	131.6
	SD	0.0	16.5	37.9	26.6
Control	M053	0.0	0.0	15.8	164.9
	M054	0.0	0.0	38.3	181.5
	M055	0.0	0.0	47.0	164.9
	M056	0.0	0.0	48.3	142.4
	M057	0.0	0.0	33.6	151.2
	MEAN	0.0	0.0	36.6	161.0
	SD	0.0	0.0	13.1	14.9

Table VIII: SRH TEST RESULTS (mm2) ANTIGEN : NEWMARKET/1/93

GROUP	FOAL	Day 0	Day 14	Day 21	Day 28
Vaccinated	M048	0.0	89.6	111.7	109.8
	M049	0.0	162.6	149	186.4
	M050	0.0	149	140.3	136
	M051	0.0	151.2	140.3	149
	M052	0.0	111.7	171.9	162.6
	MEAN	0.0	132.8	142.6	148.8
	SD	0.0	30.8	21.6	28.7
Control	M053	0.0	0.0	45.7	209.2
	M054	0.0	0.0	76.2	164.9
	M055	0.0	0.0	38.3	158.0
	M056	0.0	0.0	40.7	171.9
	M057	0.0	0.0	102.2	160.3
	MEAN	0.0	0.0	60.6	172.9
	SD	0.0	0.0	27.8	21.0

Furthermore, the total clinical scores for each group demonstrate that the vaccinated animals experienced no hyperthermia, no dyspnea no cough and with 3 out of 5 animals only with slight nasal discharge during one day. In contrast, 4 of the 5 control animals experienced significant nasal discharge, as well as dyspnea, cough and hyperthermia. The total scores for each group are depicted in table IX, and are illustrated in Figure 4.

Table IX: CLINICAL SCORES

GROUP	Gen. Condition	Anorexia	Nasal discharge	Dyspnea	Cough	Total
Vaccinated	0	0	11	0	0	11
Control	0	0	72	16	85	173

Additionally, no virus excretion was found in the vaccinated animals. In contrast, all control animals showed virus excretion ranging from 0.4 to 2.9 during the eight days after challenge. Table X contains the virus excretion results from the nasal swabs.

**Table X: VIRUS ISOLATION FROM NASAL
SWABS (log10 EID50/ml)**

GROUP	FOAL	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24
Vaccinated	M048	0	0	0	0	0	0	0	0	0	0
	M049	0	0	0	0	0	0	0	0	0	0
	M050	0	0	0	0	0	0	0	0	0	0
	M051	0	0	0	0	0	0	0	0	0	0
	M052	0	0	0	0	0	0	0	0	0	0
	MEAN	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Control	M053	0	2.5	2	2.5	3	2.5	2.5	3.5	0	0
	M054	0	0	2.5	0	2.5	1.5	2.5	0	0	0
	M055	0	3.5	3.5	2	2.5	2.5	0	0	0	0
	M056	0	1.5	4	3	3	1.5	1.5	0	0	0
	M057	2	2	2.5	2.5	3.5	3	1	0	0	0
	MEAN	0.4	1.9	2.9	2.0	2.9	2.2	1.5	0.7	0.0	0.0